Relaxin Ameliorates Renal Fibrosis and Expression of Endothelial Cell Transition Markers in Rats of Isoproterenol-Induced Heart Failure

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In the United States, heart failure affects about 6 million people, and patients with heart failure may be related to renal dysfunction. 1) Cardiorenal syndrome type 1 (CRS type 1) is a particular status defined as acute kidney injury due to a quick deterioration of cardiac function. CRS type 1 occurs in more than a quarter of people with acute decompensated heart failure and is related to prognosis. 2) This kidney dysfunction may be linked to renal fibrosis, which may play an important role in the pathogenesis of CRS type 1. 3)

Renal fibrosis leads to injured renal structure and characteristically abnormal accumulation of extracellular matrix. 4) Renal fibroblasts play a notable part in renal fibrosis, however the source of fibroblasts is still not clear and has become an obstacle. Fibroblasts produced by a significant heterogeneity of matrix have been found, as have fibroblasts of different origin, such as resident and epithelial- and endothelial-tomesenchymal transition (EndMT) fibroblasts. 5) Among these fibroblasts produced by a matrix of different origins, EndMT may be a notable source of activated fibroblasts or myofibroblasts. 6) During EndMT, endothelial cells eliminate endothelial markers, such as cluster of differentiation 31 (CD31) and vascular endothelial–cadherin, and acquire mesenchymal markers, such as fibroblast-specific protein 1 and α-smooth muscle actin (α-SMA). 5, 6) Previous studies have indicated that diabetic cardiac and renal fibrosis were related to EndMT, and inhibition of EndMT could prevent cardiac and renal fibrosis. 7, 8) Transforming growth factor-β (TGF-β) is a major promoting cytokine of renal fibrosis. In both in vivo and in vitro researches, the anti-fibrotic effect of relaxin (RLX) via downregulation of TGF-β was described. 8)

RLX is a hormone with insulin-like peptidic structure, connected by disulfide bonds with two chains. 9) RLX is produced during pregnancy in corpus luteum, and also expressed in the heart and kidneys. 10) In rats of isoproterenol (Iso)-induced myocardial injury, RLX levels in myocardium and plasma increased 3.7 and 6.9 fold respectively, and the RLX mRNA level elevated by 63% in myocardium. 9) RLX can combine to both relaxin peptide family receptor (RXFP1) (also named G protein coupled receptor 7) which is principal and RXFP2 (also named G protein coupled receptor 8). RXFP1 widely distributes in the organs including heart, arteries, kidneys. RLX may inhibit renal fibrosis through stimulating RXFP1. 10) RLX is now considered an effective anti-fibrotic hormone that prevents excessive collagen accumulation and fibrosis in diverse research models. In addition, RLX can regulate organ extracellular matrix remodeling, such as in the lungs, liver, heart, and kidneys. 11) In several in vivo animal models, the inhibitory effect of RLX on renal fibrosis has been confirmed. 8, 9) Exogenous RLX treatment may restrain the renal fibrosis that developed in RLX-knockout mice with aging. 12) RLX could directly act on diseased kidneys to prevent activation of TGF-β1. 9) An in vitro study demonstrated that RLX inhibited the renal fibroblast-to-myofibroblast transformation induced by TGF-β1. 13)

Despite the therapeutic potential of RLX in renal fibrosis, nevertheless the mechanism of its inhibitory effect on renal fibrosis is still uncertain. We investigated whether RLX affects renal dysfunction and fibrosis in rats of heart failure induced...
by isoprenaline (Iso) and examined expression of endothelial cell transition markers and TGF-β protein. We researched the possible relationship between renal fibrosis and changes in EndMT or TGF-β expression.

MATERIALS AND METHODS

Animals and Treatments A total of 50 male Sprague–Dawley rats (200–220 g; about 6 weeks old), were provided by Wenzhou Medical University Laboratory Animal Centre (Wenzhou City, China). The rats were caged and fed under standard conditions. The study was approved by the Wenzhou Medical University Animal Care and Use Ethics Committee (permission no. wydw2013-0054). All animal-handling procedures followed the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health and the guidelines of the Animal Welfare Act.

The animals were randomly divided into 5 groups (10 rats per group) for treatment: 1) control; 2) heart failure induced by treating with ISO (5 mg·kg\(^{-1}\)·d\(^{-1}\), Sigma-Aldrich, St. Louis, MO, U.S.A.) daily for 7 d; and 3) low-dose RLX (0.2 μg·kg\(^{-1}\)·d\(^{-1}\)); 4) medium-dose RLX (2 μg·kg\(^{-1}\)·d\(^{-1}\); and 5) high-dose RLX (20 μg·kg\(^{-1}\)·d\(^{-1}\)) (Peprotech, Rocky Hill, NJ, U.S.A.) daily for the initial 21 d; for the initial 7 d, RLX rats similar to the Iso-treatment group, were subcutaneously injected with Iso (5 mg·kg\(^{-1}\)·d\(^{-1}\)) to lead to heart failure, and controls were subcutaneously injected with the same amount of normal saline.

Cardiac Function Measurements We weighed the animals and then intraperitoneally injected 1% pentobarbital sodium (40 mg·kg\(^{-1}\)) to anaesthetise. We inserted a catheter into the left ventricle (LV) via carotid artery to monitor changes in LV end diastolic pressure (LVEDP), LV mean systolic pressure (LVSP), and maximum change rate of LV pressure (+dP/dt\(_{\text{max}}\), −dP/dt\(_{\text{max}}\)).

Renal Function, Cardiac and Renal Weight Indices We euthanized the rats humanely, drew the blood to monitor the levels of serum creatinine (SCr) and blood urea nitrogen (BUN) with an 7180 automatic biochemical analyzer (Hitachi High-Technologies Corp., Japan), and infused the left ventricle with precooled saline (4°C) until the heart and kidneys eviscerated. We quickly excised, washed and weighed the heart, and then separated out left and right ventricles from the heart and weighed. The left and right ventricle weight indices (LVWI and RVWI) were computed as free wall mass (mg) of the left and right ventricles/body mass (g), respectively. The kidney weight index (KWI) was computed as the mean mass (mg) of the two kidneys/body mass (g).

Haematoxylin–Eosin (H&E) Staining We embedded tissues of the left ventricular cardiac apex and kidneys in paraffin and sliced those tissues into pieces as routine. We stained paraffin sections (4-μm thick) with haematoxylin and eosin, inspected with light microscopy and imaged at ×400 and ×200 magnification in Figs. 1A and 2; ×400 magnification in Fig. 1B.

Masson Trichrome Staining We stained paraffin sections with Masson trichrome. Cell nucleus was stained black, cytoplasm red, and fibrous tissues blue. We examined the sections with light microscopy and photographed at ×40 and ×200 magnification. We randomly chose five non-repeated visual fields of one section, magnification ×200. We measured collagen areas of heart and kidneys with Image-Pro Plus (Media Cybernetics, Rockville, MD, U.S.A.), and averaged the collagen amount of every area.

Analysis of Collagen Type I and III by Enzyme-Linked Immunoassay (ELISA) We sliced tissues of LV myocardium and kidneys (100 mg) into pieces, and pulivered those tissues in 1 mL phosphate buffered saline (pH 7.4) on ice. After centrifugated at 1000×g for 20 min, the supernatant liquid was obtained and the amount of collagen types I and III was examined with an ELISA kit (Shanghai Boyun Biotech, China).

Immunofluorescence Assay of Renal Tissues Renal sections underwent dual immunofluorescence staining. Endogenous peroxidase activity of renal sections was blocked by treating with 3% methanol–H\(_2\)O\(_2\), and non-specific sites were blocked with 10% fetal bovine serum. We incubated the sections with primary mixed antibodies for anti-α-SMA (Catalog Number BM0002, Wuhanboshi, China, 1:100) or anti-CD31 (Catalog Number SC-1505-R, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A., 1:30) overnight at 4°C, then antibodies for DyLight 594 AffiniPure goat anti-rabbit immunoglobulin G (IgG) [H+L] and DyLight 488 AffiniPure goat anti-mouse IgG [H+L] (EarthOX, Millbrae, CA, U.S.A., 1:300). After eluting, 4,6-diamidino-2-phenylindole hydrochloride (DAPI) staining solution (Beijing Leagene Biotechnology, China) was mixed and eluted. Sections were photographed, magnification ×200. The outcomes were measured with fluorescence microscopy (Nikon Corp., Japan) and managed with Image-Pro Plus (Media Cybernetics, U.S.A.). We incubated control sections with phosphate buffered saline only.

Western Blot Analysis of Renal Tissues We mixed a slice of renal tissue (100 mg) homogeneously with 10 μL phenylmethylsulfonyl fluoride (100 mmol/L) and 1 mL radio immunoprecipitation assay lysis buffer. We centrifuged the mixture at 12000×g for 10 min at 4°C and obtained supernatant liquid. We detected the concentrations of protein with a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, China). The same quantities of samples (50 μg) were run on sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Beyotime Institute of Biotechnology, China), which were blocked with 5% skim milk for 1 h and incubated with primary antibodies (anti-α-SMA, Catalog Number BM0002, Wuhanboshi, China, 1:1000; anti-CD31, Santa Cruz Biotechnology, U.S.A., 1:1000; anti-TGF-β1, Catalog Number BS1361, Bioworld Technology, China, 1:1000) at 4°C overnight. Immunoreactive bands were revealed by chemiluminescent horseradish peroxidase substrate (Applygen Technologies, China), and scans were acquired by the Bio-Rad gel image analysis system (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.) and managed with Image-Pro Plus (Media Cybernetics, Inc.).

Statistical Analysis Data are expressed as mean±standard deviation (S.D.). Data were analyzed by Student’s t-test for comparing the two groups or one-way ANOVA, followed by least significant difference test for multi-group comparisons. All statistical analyses involved use of SPSS 16.0 (SPSS Inc., Chicago, IL, U.S.A.). p<0.05 was considered statistically significant.
RESULTS

**Effect of RLX on Cardiac Function of Rats** As compared to controls, Iso subcutaneous injection in rats led to cardiac fibrosis, with significantly reduced heart rate, LVSP, \(+\frac{dp}{dt}\text{max}\) and increased LVEDP, \(-\frac{dp}{dt}\text{max}\) \((p<0.01, \text{Table 1})\). As compared with Iso alone, treatment with RLX weakened these changes of cardiac function induced by Iso, with significantly increased heart rate, LVSP, \(+\frac{dp}{dt}\text{max}\) and reduced LVEDP, \(-\frac{dp}{dt}\text{max}\) \((p<0.05)\).

**Effect of RLX on Renal Function, Cardiac and Renal Weight Indices of Rats** Changes of SCr and BUN levels are not significant with Iso treatment as compared with controls, and treatment with RLX as compared with Iso alone. As compared with controls, LVWI, RVWI and KWI were significantly increased and body weight was reduced with Iso treatment \((p<0.01, \text{Table 2})\); as compared with Iso alone, treatment with RLX reduced LVWI, RVWI and KWI \((p<0.05)\).

**Histopathological Observations of Cardiac and Renal Tissues in Rats** Cardiac tissues in rats with Iso treatment showed universal accumulation of fibrous tissue, myocardial hypertrophy, injured myocardial structure, leukocyte in-
IsoSCr, serum creatinine; BUN, blood urea nitrogen; KWI, kidney weight index.

Amount of collagen types I and III in myocardial tissue and renal tissue homogenate was greater in rats with Iso treatment than controls (p < 0.01, Table 3). RLX treatment significantly reduced the amount as compared with Iso alone (p < 0.01).

**Effect of RLX on α-SMA and CD31 Protein Levels in Renal Tissues of Rats** On immunofluorescence assay and Western blot analyses, as compared with controls, Iso increased α-SMA protein level and decreased CD31 protein level in renal tissues (p < 0.01). As compared with Iso alone, RLX treatment reversed the protein level changes (Figs. 3A–D).

**Effect of RLX on TGF-β1 Protein Level in Renal Tissues of Rats** The endogenous TGF-β1 expression in renal tissues was greater with Iso treatment than controls (p < 0.01). As compared with Iso alone, RLX treatment lowered the TGF-β1 protein level (Fig. 3E).

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**Table 2. Effect of Relaxin (RLX) on Indices of Cardiac Weight, Renal Function and Weight**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BW (g)</th>
<th>LVWI (μg·g⁻¹)</th>
<th>RVWI (μg·g⁻¹)</th>
<th>SCr (μmol·L⁻¹)</th>
<th>BUN (mmol·L⁻¹)</th>
<th>KWI (mg·g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>269±9.9</td>
<td>2.47±0.15</td>
<td>0.62±0.07</td>
<td>6.32±1.73</td>
<td>32.70±9.64</td>
<td>4.11±0.12</td>
</tr>
<tr>
<td>Iso</td>
<td>228±8.1**</td>
<td>3.25±0.22**</td>
<td>0.84±0.09**</td>
<td>7.65±1.82</td>
<td>36.50±8.02</td>
<td>4.75±0.10**</td>
</tr>
<tr>
<td>Iso+low-dose RLX</td>
<td>240±22.4</td>
<td>3.09±0.18</td>
<td>0.76±0.10*</td>
<td>8.07±1.07</td>
<td>36.80±8.36</td>
<td>4.62±0.24</td>
</tr>
<tr>
<td>Iso+medium-dose RLX</td>
<td>242±8.0#</td>
<td>2.78±0.12**</td>
<td>0.74±0.09#</td>
<td>6.36±1.56</td>
<td>30.20±7.00</td>
<td>4.45±0.18**</td>
</tr>
<tr>
<td>Iso+high-dose RLX</td>
<td>256±16.0##</td>
<td>2.53±0.32**</td>
<td>0.65±0.08##</td>
<td>8.36±1.36</td>
<td>31.00±6.53</td>
<td>4.28±0.24##</td>
</tr>
</tbody>
</table>

Data are mean±S.D. (n=10). **p<0.01 vs. control; *p<0.05, ***p<0.01 vs. Iso. BW, body weight; LVWI, left ventricle weight index; RVWI, right ventricle weight index; SCr, serum creatinine; BUN, blood urea nitrogen; KWI, kidney weight index.

**Table 3. Effect of Relaxin (RLX) on the Expression of Collagen Types I and III in Cardiac and Renal Tissues of Rat**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Collagen type I (ng·mL⁻¹)</th>
<th>Collagen type III (ng·mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rats</td>
<td>Cardiac tissue</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>2.20±0.66</td>
</tr>
<tr>
<td>Iso</td>
<td>9</td>
<td>9.17±0.97**</td>
</tr>
<tr>
<td>Iso+low-dose RLX</td>
<td>8</td>
<td>7.75±1.16##</td>
</tr>
<tr>
<td>Iso+medium-dose RLX</td>
<td>8</td>
<td>5.62±0.55#</td>
</tr>
<tr>
<td>Iso+high-dose RLX</td>
<td>8</td>
<td>2.81±0.77##</td>
</tr>
</tbody>
</table>

Data are mean±S.D. **p<0.01 vs. control; *p<0.05, ***p<0.01 vs. Iso.
DISCUSSION

In this study, heart function was impaired, renal structure was injured, collagen accumulation indicating renal fibrosis was severe and expression of TGF-β and α-SMA in renal tissues was increased with Iso treatment versus control treatment in rats, which probably indicated that renal EndMT was induced. With RLX treatment, cardiac function was improved, and renal fibrosis and expression of TGF-β and α-SMA in renal tissues were reduced, which probably indicated inhibited renal EndMT with RLX treatment.

CRS type 1 is defined as acute kidney injury resulted from an acute heart disorder (including acute myocardial infarction). In patients of acute heart failure, including those with acute heart disorder (including acute myocardial infarction) and renal EndMT with RLX treatment.

In renal fibrosis, fibroblasts are notable effector cells. Fibroblasts exist in the extracellular matrix of connective tissues with specific properties of mesenchymal cells, which may produce collagen proteins to constitute the extracellular matrix. Renal fibrosis is chiefly generated by resident fibroblasts, nevertheless activated fibroblasts may originate from transdifferentiation of renal resident cells such as endothelial cells, called EndMT. EndMT was previously considered a crucial procedure in cardiac development, but EndMT is now considered to also occur postnatally in diverse pathologic settings, involving tumour development, pulmonary hypertension, cardiac fibrosis and renal fibrosis. EndMT occurs in approximately 10% of emerging fibroblasts during renal fibrosis, then leading to renal fibrosis. During EndMT, resident endothelial cells take on a mesenchymal phenotype, characterized by loss of cell–cell junctions, the acquisition of migratory and invasive characteristics, loss of endothelial markers such as CD31 and acquisition of mesenchymal markers such as α-SMA or fibroblast-specific protein 1. Inhibition of EndMT may be effective therapy for preventing the development of renal fibrosis.

RLX, a insulin-like peptidic hormone, has been generally recognized for its function in pregnancy, and current research has investigated the beneficial effects of this hormone in cardiovascular diseases and fibrosis. RLX could inhibit cardiac fibrosis via TGF-β-induced EndMT by Notch-mediated signaling in ischemic-associated rat cardiac fibrosis. In primary cultures of neonatal cardiac stromal cells, RLX may take its widely-known anti-fibrotic effect by inhibiting TGF-β-stimulated cardiac fibroblast–myofibroblast transition. The protective effect of RLX on the kidney has been verified in diverse experimental models. Knockout of mouse RLX gene leads to renal hypertrophy, dysfunction and fibrosis, and exogenous RLX treatment reversed the glomerular sclerosis and tubulointerstitial fibrosis. Furthermore, structural damage and decreased renal function established in aged rats were reversed with RLX treatment. The antifibrotic effects of RLX on the kidney also have been described in models of papillary necrosis, angiotensin II-induced hypertension, renal mass reduction, and anti-glomerular basement membrane disease. In the kidneys of humans and rats, RXFP1 mRNA has been detected. It is localized the expression of RXFP1 by immunohistochemical analyses in the mesangial cells, inner medullary collecting ducts and proximal tubules. RLX and RXFP1 are both expressed in small renal arteries of rats and mice. In rat renal myofibroblasts, RLX stimulates RXFP1 to activate G proteins, leading to phosphorylation of extracellular regulated protein kinases and increased expression of nitric oxide synthase (NOS1, neuronal NOS). Expression of NOS1 and nitric oxide (NO)-cGMP signaling result in NO production inhibiting TGF-β signaling, consequently preventing myofibroblast differentiation and collagen synthesis. Besides, NO production reduced fibronectin levels induced by TGF-β and increased fibronectin degradation. Therefore, targeting the RLX pathway may be a potential therapeutic method in treating renal fibrotic diseases.

In this study, Iso-induced cardiac and renal fibrosis was ameliorated by RLX treatment, and the effect was even greater with high-dose RLX (20 vs. 2 μg·kg⁻¹·d⁻¹, and 2 vs. 0.2 μg·kg⁻¹·d⁻¹), so the anti-fibrotic effect of RLX may be dose-related. Iso-treated rats showed increased expression of α-SMA and accumulation of collagen type I and III and reduced expression of CD31 in renal tissues as compared with controls, which suggests that endothelial characteristics were lost and the fibroblast property was amplified, for the possible manifestation of EndMT in renal fibrosis. Also as the key mediator of EndMT, the level of TGF-β in renal tissues was increased in Iso-treated rats. These effects were weakened by RLX treatment, so RLX may probably inhibit renal fibrosis by preventing EndMT.

Neurohormonal activation may play an important part in the process of renal fibrosis, and various cytokines and growth factors involve in this procedure. TGF-β has been considered a key promoter of renal fibrosis. TGF-β1 and TGF-β2 can promote myofibroblast differentiation and matrix synthesis while diminishing matrix degradation by regulating the activity of matrix metalloproteinases. TGF-β1 can induce endothelial cell proliferation and accelerate EndMT. In mouse primary cultures of kidney endothelial cells, TGF-β1 time- and dose-dependently induced α-SMA expression along with loss of CD31 and vascular endothelial–cadherin expression. Disturbed TGF-β activity results in a series of downstream consequences involving the prevention of myofibroblast differentiation, abolition of extracellular matrix synthesis, and intensified extracellular matrix degradation induced by matrix metalloproteinases. TGF-β may be a key factor in EndMT.
with renal fibrosis. The effect of RLX on inhibiting renal fibrosis may be via inhibiting renal EndMT probably, and inhibiting TGF-β protein expression may be a key pathway.

RLX has antifibrotic characteristics in various organs, including the kidney. Endogenous RLX can prevent progression of tubulointerstitial fibrosis in the injured mouse kidney, mainly by preventing renal myofibroblast differentiation in vivo. In cultured human mesangial cells, exogenous RLX significantly inhibited high glucose-induced expression of fibronectin, collagen, α-SMA, and TGF-β1 mRNA, so the inhibitory effect of RLX on excessive extracellular matrix production may be by inhibiting the overexpression of TGF-β1 mRNA. In conclusion, RLX may ameliorate renal fibrosis in rats of Iso-induced heart failure, and it is inferred that prevention of the EndMT may be one of the possible potential signaling pathways. Nevertheless, the decreased α-SMA and increased CD31 with RLX treatment show only that RLX can prevent fibrosis with ameliorating the expression the endothelial markers. From these data, the EndMT cannot be ascertained definitely. However, more specific markers of renal fibrosis could be considered to solve these problems. In addition, further studies should identify the effect of RLX on preventing renal fibrosis and explore the role of inhibiting renal EndMT in this process.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


